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(54) Title: METHODS FOR ASSESSING EFFICACY OF CHEMOTHERAPEUTIC AGENTS

(57) Abstract: Methods are provided for accurately predicting efficacy of chemotherapeutic agents. Methods of the invention increase the positive predictive value of chemosensitivity assays by assessing both the ability of a chemotherapeutic to destroy cells and the genetic propensity of those cells for resistance. Results obtained using methods of the invention provide insight into the in vivo effectiveness of a therapeutic, and lead to more effective chemotherapeutic treatment.

METHODS FOR ASSESSING EFFICACY OF CHEMOTHERAPEUTIC AGENTS

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Related Applications

[0001] This application claims priority to U.S. Application Serial No. 60/417,439, filed October 12, 2002, and U.S. Application Serial No. 10/336,659, filed January 2, 2003, the disclosures of which are incorporated by reference herein.

10

Field Of The Invention

[0002] The invention relates to methods for assessing efficacy of chemotherapeutic agents.

Background

[0003] Cancer chemotherapy involves the use of cytotoxic drugs to destroy unwanted cells in patients. Treatment may consist of using one or more cytotoxic drugs, depending on the nature of the disease being treated. However, drug toxicity and drug resistance are significant barriers effective chemotherapy.

[0004] Toxicity from chemotherapeutic agents produces side effects ranging from mild trauma to death. Moreover, repeated exposure to chemotherapeutic drugs is itself often fatal. As chemotherapeutic drugs are carried in the blood, they are taken up by proliferating cells, including normal cells. Tissues with high growth rates such as bone marrow and epithelial tissues, including the gastrointestinal tract, are normally most susceptible to toxic side effects. Some drugs have additional toxic effects on other tissues, such as the urinary tract, myocardium, or pancreas. Chemotherapeutic agents may cause direct injury to the heart, either acutely, in the form of myocardial tissue injury or dysrhythmias, or in a delayed or chronic fashion associated with congestive heart failure.

[0005] Target cells, such as malignant or diseased cells, may be intrinsically resistant to chemotherapeutic drugs or they may acquire resistance as a result of exposure. A target cell may be genetically predisposed to resistance to particular chemotherapeutics. Alternatively, the cell may not have receptors or activating enzymes for the drug or may not be reliant on the biochemical process with which the drug interferes.

[0006] Acquired resistance also may develop after cells have been exposed to a drug or to similar classes of drugs. One example of acquired drug resistance is the multiple drug resistance

phenotype. Multiple drug resistance is a phenomenon of cross-resistance of cells to a variety of chemotherapeutic agents which are not structurally or functionally related. This phenomenon is typically mediated by p-glycoprotein, a cell membrane pump that is present normally on the surface of some epithelial cells. The protein actively removes drug from the cell, making it resistant to drugs that are substrates for the cell membrane pump.

[0007] A critical issue in cancer chemotherapy is the ability to select drugs that not only affect cancer cell phenotype in cell culture assays, but are also not subject to resistance. The present invention addresses that issue.

10

Summary Of The Invention

[0008] The invention provides methods for accurately predicting efficacy of chemotherapeutic agents. Methods of the invention increase the positive predictive value of chemosensitivity assays by assessing both the ability of a chemotherapeutic to affect tumor cells phenotype and the genetic propensity of the patient for resistance to the chemotherapeutic. Results obtained using methods of the invention provide insight into the *in vivo* effectiveness of a therapeutic, and lead to more effective, individualized, chemotherapeutic choices.

[0009] According to the invention, a phenotype assay screens a therapeutic candidate for the ability to affect the phenotype of tumor cells in culture. A therapeutic candidate that produces the desired phenotypic effect (e.g., cell death, decreased motility, changes in cellular adhesion, angiogenesis, or gene expression, among others) then is screened against genetic properties of cells of the patient which make resistance to the therapeutic candidate likely or possible. A therapeutic candidate that has a desired phenotypic effect on patient tumor cells and that does not appear to be subject to genetic-based resistance is selected for use. As a result of combining phenotypic and genetic data, use of the invention increases the likelihood that a therapeutic candidate, chosen on the basis of its ability to affect cellular phenotype, will be effective when administered to patients.

[0010] Accordingly, the invention provides methods for assessing efficacy of chemotherapeutic agents comprising exposing cells to a chemotherapeutic agent, conducting an assay to determine whether the chemotherapeutic agent affects tumor cell phenotype, and identifying genetic characteristics of cells of the patient (which may or may not be tumor cells) that indicate a propensity for resistance to the chemotherapeutic agent.

[0011] In a preferred embodiment, a phenotypic assay for use in the invention comprises obtaining a tumor explant from a patient, culturing portions of the explant, growing a monolayer of relevant cells from the explant, exposing the monolayer to a drug candidate, and assessing the

ability of the drug candidate to alter tumor cell phenotype. A preferred phenotypic assay is disclosed in U.S. Patent No. 5,728,541, and in co-owned, co-pending U.S. Application Serial No. 10/208,480, both of which are incorporated by reference herein.

[0012] Genotype analysis according to the invention is accomplished by any known method.

5 A preferred method comprises comparing the genotype, or portion thereof, of cells obtained from the patient with genotypes known to be associated with drug resistance generally, or specifically with respect to a therapeutic candidate being evaluated. For example, the existence in patient cells of a polymorphic variant that is known or suspected to confer resistance to a therapeutic candidate would screen that candidate out as a potential therapeutic against those cells. Genetic
10 characteristics of patient cells are determined by methods known in the art (e.g., sequencing, polymorphisms) as set forth below. The impact of a patient's genotype upon drug resistance may be determined by reference to genetic databases or libraries that catalog known mutations or polymorphisms related to resistance.

[0013] The present invention also provides methods for selecting a chemotherapeutic agent
15 for treating a patient based on results obtained from the phenotypic and genotypic assays. In a preferred embodiment, the present invention allows for the assessment of whether a chemotherapeutic agent will be effective in treating a cancer when administered to a patient. According to the invention, chemotherapeutic agents or combinations of chemotherapeutic agents are selected for treatment where an effect on cellular phenotype is observed and
20 characteristics of genetic-based resistance are not observed.

[0014] Methods of the invention are useful in drug or chemotherapeutic agent screening to provide information indicative of the *in vivo* reactivity of the cells, and thus the specific efficacy as to a particular patient. Methods of the invention are also useful to screen new drug candidates for therapeutic efficacy and to provide a basis for categorizing drugs with respect to the tumor
25 types against which they will work best.

[0015] A phenotypic assay according to the invention is conducted on cells obtained from a tumor explant from a patient. Genotypic assays of the invention are performed on genetic data obtained from patient cells, regardless of their source. Thus, a genotypic assay can be performed on somatic cells obtained from the patient or on cells from the same tumor that is evaluated in
30 the phenotypic assay. Assays of the invention can be performed on an individualized basis or on a pool of samples obtained from multiple individual patients. If assays are conducted on pooled samples, the phenotypic characteristics of the pool of samples are determined followed by individualized genotypic assays on specific patients. This allows multiplexing of the phenotypic portion of the assay.

Detailed Description Of The Invention

[0016] This invention provides methods for assessing efficacy of chemotherapeutic agents. Specifically, the invention provides methods for assessing the efficacy of chemotherapeutic agents based on phenotypic changes observed in tumor cells obtained from a patient and genetic characteristics of the patient that indicate general or specific chemotherapeutic resistance. In one aspect of the invention, efficacy of a chemotherapeutic agent is assessed based upon the results of the phenotypic and genotypic assays. In another aspect of the invention, chemotherapeutic agents are selected for treating a patient based on the results of the phenotypic and genotypic assays.

[0017] The present invention is also useful for screening of therapeutic agents against other diseases, including but not limited to, hyperproliferative diseases, such as psoriasis. In addition, the screening of agents that retard cell growth (anti-cancer, anti-hyperproliferative), including agents that enhance or subdue intracellular biochemical functions, are evaluated using methods of the present invention. For example, the effects of therapeutics on the enzymatic processes, neurotransmitters, and biochemical pathways are screened using methods of the invention. Methods of the invention can be practiced on any type of cell obtained from a patient, including, but not limited to, normal somatic cells, malignant cells, abnormal proliferating cells, and other diseased cells. Cells are obtained from any patient sample, including, but not limited to, blood samples and buccal smears. The skilled artisan recognizes that methods of the invention can be practiced using a variety of different samples.

[0018] In one step of the invention, a phenotype assay is employed to assess sensitivity and resistance to chemotherapeutic agents. The phenotypic assay is performed *in vitro* using cultured cells. The phenotype assay allows for identification and separation of target cells from other cells found in a tissue sample, as well as direct measurement and monitoring of target cells in response to chemotherapeutic treatment. Direct measurements and monitoring of live cells are performed using known methods in the art including, for example, the measuring of doubling rate, fraction proliferative assays, monitoring of cytostasis, cell death, cell adhesion, gene expression, angiogenesis, cell motility, and others. Direct measurements also include known assays, such as those directed to measurement and monitoring of apoptosis, senescence, and necrosis.

[0019] In another step of the invention, a genotype assay is performed to determine whether cells from a patient comprise a genetic characteristic associated with resistance to the chemotherapeutic agents. Genotype assays reveal latent resistance to chemotherapeutic agents

not observed by phenotypic assays. Genotypic assays may measure characteristics, such as metabolism, toxic effects, absorption of a therapeutic candidate.

[0020] In one embodiment of the invention, the phenotypic assay is performed using cell culture monolayers prepared from tumor cells. In a preferred embodiment, monolayers are cultured from cohesive multicellular particulates generated from a tumor biopsy. Explants of tumor tissue sample are prepared non-enzymatically, for initial tissue culture monolayer preparation. The multicellular tissue explant is removed from the culture growth medium at a predetermined time to both allow for the growth of target cells and prevent substantial growth of non-target cells such as fibroblasts or stromal cells.

[0021] By way of example, in one embodiment of the invention, a cell culture monolayer is prepared in accordance with the invention using the following procedure. A biopsy of non-necrotic, non-contaminated tissue is obtained from a patient by any suitable biopsy or surgical procedure known in the art. In a preferred embodiment, the tissue sample is tumor tissue. The size of the biopsy sample is not central to the methods provided herein, but a sample is preferably about 5 to 500 mg, and more preferably about 100 mg. Biopsy sample preparation generally proceeds under sterile conditions. Cohesive multicellular particulates (explants) are prepared from the tissue sample by enzymatic digestion or mechanical fragmentation. Ideally, mechanical fragmentation of the explant occurs in a medium substantially free of enzymes that are capable of digesting the explant. For example, the tissue sample may be minced with sterile scissors to prepare the explants. In a particularly preferred embodiment, the tissue sample is systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion creates smooth cut edges on the resulting tissue multicellular particulates. After the tissue sample has been minced, the particles are plated in culture flasks (for example, 9 explants per T-25 flask or 20 particulates per T-75 flask). The explants are preferably evenly distributed across the bottom surface of the flask, followed by initial inversion for about 10-15 minutes. The flask is then be placed in a non-inverted position in a 37° C CO₂ incubator for about 5-10 minutes. In another embodiment in which the tissue sample comprises brain cells, the flasks are placed in a 35° C, non-CO₂ incubator. Flasks are checked regularly for growth and contamination.

[0022] The multicellular explant is removed from the cell culture at a predetermined time, as described below. Over a period of a few weeks a monolayer is produced. With respect to the culturing of tumor cells, it is believed (without any intention of being bound by the theory) that tumor cells grow out from the multicellular explant prior to contaminating stromal cells. Therefore, by initially maintaining the tissue cells within the explant and removing the explant at

a predetermined time, growth of the tumor cells (as opposed to stromal cells) into a monolayer is facilitated. The use of the above procedure to form a cell culture monolayer maximizes the growth of tumor cells from the tissue sample, and thus optimizes the phenotypic and genotypic assays.

5 [0023] Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to oversee growth of the monolayer and ascertain the time to initiate the phenotypic assay. Prior to the phenotypic assay, monitoring of the growth of cells may be conducted by visual monitoring of the flasks on a periodic basis, without killing or staining the cells and without removing any cells from the culture flask. Data from periodic
10 counting or measuring is then used to determine growth rates or cell motility, respectively.

[0024] Phenotypic assays are performed on cultured cells using a chemotherapeutic drug response assay with clinically relevant dose concentrations and exposure times. One embodiment of the present invention contemplates a phenotypic assay that assesses whether chemotherapeutic agents effect cell growth. Monolayer growth rate is monitored using, for
15 example, a phase-contrast inverted microscope. In one embodiment, culture flasks are incubated in a (5% CO₂) incubator at about 37° C. The flask is placed under the phase-contrast inverted microscope, and ten fields (areas on a grid inherent to the flask) are examined using a 10X objective. In general, the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell
20 occupancy for each field examined is noted, and averaging of these percentages then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Alternatively, confluency
25 is judged independently for each flask. Monolayer cultures may be photographed to document cell morphology and culture growth patterns. The applicable formula is:

$$\text{Percent confluency} = \frac{\text{estimate of the area occupied by cells}}{\text{total area in an observed field}}$$

As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area
30 of the field is 100%, percent confluency is 30/100, or 30%.

[0025] Following initial culturing of the multicellular tissue explant, the tissue explant is removed from the growth medium at a predetermined time. In one embodiment, the explant is removed from the growth medium prior to the emergence of a substantial number of stromal cells from the explant. Alternatively, the explant may be removed according to the percent

confluency of the cell culture. In one embodiment of the invention, the explant is removed at about 10 to about 50 percent confluency. In a preferred embodiment of the invention, the explant is removed at about 15 to about 25 percent confluency. In a particularly preferred embodiment, the explant is removed at about 20 percent confluency. By removing the explant in either of the above manners, a cell culture monolayer predominantly composed of target cells (e.g., tumor cells) is produced. In turn, a substantial number of non-target cells, such as fibroblasts or other stromal cells, fail to grow within the culture. Ultimately, this method of culturing a multicellular tissue explant and subsequently removing the explant at a predetermined time allows for increased efficiency in both the preparation of cell cultures and subsequent phenotypic and genotypic assays for assessing efficacy of chemotherapeutic agents.

[0026] In another embodiment, a phenotypic assay assesses whether chemotherapeutic agents effect cell motility. Methods for measuring cell motility are known by persons skilled in the art. Generally, these methods monitor and record the changes in cell position over time. Examples of such methods include, but are not limited to, video microscopy, optical motility scanning (for example, see U.S. Pat. No. 6,238,874, the disclosure of which is incorporated by reference herein) and impedance assays. In a preferred embodiment, cell motility assays are carried out using monolayer cultures of malignant cells as described herein.

[0027] Cell culture methods of the invention permit the expansion of a population of proliferating cells in a mixed population of abnormal proliferating cells and other (normal) cells. The mixed population of cells typically is a biopsy or sample from a solid tumor. A tissue sample from the patient is harvested, cultured and analyzed for genetic indicia of resistance to chemotherapeutics. Subcultures of the cells produced by the culture methods described above may be separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment for the patient. By way of example, procedures for culturing the malignant cells and determining a phenotypic to a chemotherapeutic agent may be performed in the following manner. First, a specimen is finely minced and tumor fragments are plated into tissue culture. The cells are then exposed to growth medium, such as a tumor-type defined media with serum. The cells are trypsinized, preferably, but not necessarily, when greater than 150,000 cells grown out from tumor fragment. The cells are preferably plated into a Terasaki plate at 350 cells per well. The cells are analyzed to verify that a majority of cells are tumor epithelial cells. Non-adherent cells are removed from the wells. The cells are treated with 6 concentrations and 2 control lanes of chemotherapeutic agent or agents for preferably 2 to 4 hours. The chemotherapeutic agents are removed by washing. The cells are incubated for

preferably 3 days. The living cells are counted to calculate the kill dose that reduces by 40% the number of cells per well from control wells.

5 [0028] The culture techniques of the present invention result in a monolayer of cells that express cellular markers, secreted factors and tumor antigens in a manner representative of their expression *in vivo*. Specific method innovations such as tissue sample preparation techniques render this method practically, as well as theoretically, useful.

[0029] According to the present invention, cells from a patient are analyzed for genetic characteristics (abnormalities) specific to a patient. Genetic characteristic of a cell or cell population can be analyzed alone or in combination with other characteristics. Genetic
10 characteristics of the invention can be, without limitation, a genetic polymorphism or a mutation, such as an insertion, inversion, deletion, or substitution. In one embodiment, nucleic acids are isolated from cells of a patient and analyzed to identify genotypic characteristics of the cells. The isolated nucleic acid is DNA or RNA. The nucleic acid, preferably, is analyzed in a
15 microarray for DNA-encoded polymorphisms in the coding or control regions of the gene. In another embodiment, the nucleic acid is analyzed in a microarray for aberrant expression of one or more genes. In this embodiment, the microarray contains nucleic acids that are characteristic of known malignancies, as well as nucleic acids, that are not correlated with known malignancies so that previously unknown relationships between gene expression and a proliferative disease or condition may be identified.

20 [0030] A preferred method of the invention comprises comparing the genotype, or portion thereof, of cells from a patient with genotypes known to be associated with drug resistance generally, or specifically with respect to a therapeutic candidate being evaluated. For example, the existence in patient cells of a polymorphic variant that is known or suspected to confer resistance to a therapeutic candidate would screen that candidate out as a potential therapeutic
25 against those cells.

[0031] Methods for isolating and analyzing nucleic acids derived from the cells are known in the art. The presence of known proliferation markers, such as the aberrant expression of one or more genes, the epidermal growth factor receptor (EGFR) cyclin D1, p16cyclin-kinase inhibitor, retinoblastoma (Rb), transforming Growth Factor β (TGF β) receptor/smad, MDM2 or p53
30 genes, may be determined by, for example, northern blotting or quantitative polymerase chain reaction (PCR) methods (i.e., RT-PCR).

[0032] In one embodiment of the present invention, mRNA (polyA⁺ mRNA) is isolated and labeled cDNA is prepared therefrom. The labeled cDNA is prepared by synthesizing a first strand cDNA using an oligo-dT primer, reverse transcriptase and labeled deoxynucleotides, such

as, Cy5-dUTP, commercially available from Amersham Pharmacia Biotech. Radio-labeled nucleotides also can be used to prepare cDNA probes. The labeled cDNA is hybridized to the microarray under sufficiently stringent conditions to ensure specificity of hybridization of the labeled cDNA to the array DNA.

5 [0033] In another embodiment of the invention, the labeled array is visualized. Visualization of the array may be conducted in a variety of ways. For instance, when the reading of the microarray is automated and the labeled DNA is labeled with a fluorescent nucleotide, the intensity of fluorescence for each discrete DNA of the microarray can be measured automatically by a robotic device that includes a light source capable of inducing fluorescence of the labeled
10 cDNA and a spectrophotometer for reading the intensity of the fluorescence for each discrete location in the microarray. The intensity of the fluorescence for each DNA sample in the microarray typically is directly proportional to the quantity of the corresponding species of mRNA in the cells from which the mRNA is isolated. It is possible to label cDNA from two cell types (i.e., normal and diseased proliferating cells) and hybridize equivalent amounts of both
15 probe populations to a single microarray to identify differences in RNA expression for both normal and diseased proliferating cells. Tools for automating preparation and analysis of microarray assays, such as robotic microarrayers and readers, are available commercially from companies such as Gene Logic and Nanogen and are under development by the NHGRI. The automation of the microarray analytical process is desirable and, for all practical purposes
20 necessary, due to the huge number and small size of discrete sites on the microarray that must be analyzed.

[0034] In a further embodiment, DNA microarrays are used in combination with the cell culturing method of the present invention due to the increased sensitivity of mRNA quantification protocols when a substantially pure population of cells are used. For their ease of
25 use and their ability to generate large amounts of data, microarrays are preferred, when practicable. However, certain other or additional qualitative assays may be preferred in order to identify certain markers.

[0035] In another embodiment, the presence of, or absence of, specific RNA or DNA species are identified by PCR procedures. Known genetic polymorphisms, translocations, or insertions
30 (i.e., retroviral insertions or the insertion of mobile elements, such as transposons) often can be identified by conducting PCR reactions with DNA isolated from cells cultured by the methods of the present invention. Where the sequence anomalies are located in exons, the genetic polymorphisms may be identified by conducting a PCR reaction using a cDNA template. Aberrant splicing of RNA precursors also may be identified by conducting a PCR reaction using

a cDNA template. An expressed translocated sequence may be identified in a microarray assay, such as the Affymetrix p53 assay.

[0036] In one embodiment, small or single nucleotide substitutions are identified by the direct sequencing of a given gene by the use of gene-specific oligonucleotides as sequencing
5 primers. In a further embodiment, single nucleotide mutations are identified through the use of allelic discrimination molecular beacon probes, such as those described in Tyagi, S. and Kromer, F.R. (1996) *Nature Biotech.* 14:303-308 and in Tyagi, S. et al., (1998) *Nature Biotech.* 16:49-53, the disclosures of each of which are incorporated by reference herein.

[0037] Genotypic analysis may be based on experimentation or experience. Sources for such
10 empirical data made be obtained from, but not limited to clinical records and/or animal tumor transplant studies. Genetic characteristics found in the patient cells can be compared to a database containing known tumor genotypes and their respective resistance to chemotherapeutic agents. In a preferred embodiment, a database containing genotypes and their respective drug resistance profile is used to compare genotypic characteristics of the target cells to resistance to
15 chemotherapeutic agents *in vivo*. Computer algorithms are useful for carrying out pattern matching routines in complex systems, such as genetic data-mining. A linear regression algorithm, for example, can be utilized to analyze a database and identify the genotype most closely matching the genetic characteristics in the patient cells. In one embodiment, a comparative analysis of genotypes is performed using a known linear regression algorithm.

[0038] According to the invention, genotypic characteristics of patient cells are analyzed to
20 establish whether such characteristics are associated with resistance to chemotherapeutic agents *in vivo*. While the above-mentioned genotypic assays are useful in the analysis of nucleic acids derived from cells produced by the culture methods embodied in the present invention, numerous additional methods are known in the general fields of molecular biology and molecular
25 diagnostics that may be used in place of the above-referenced methods. Information obtained from genotypic assays is analyzed to determine efficacy of chemotherapeutic agents.

[0039] In a further embodiment of the invention, data obtained by practicing the methods of the invention, including phenotypic, genotypic and patient outcome information, is stored in
30 databases. The contents of these databases include, but are not limited to, observed *in vitro* phenotypes (disease factors) and genotypes (host factors). By applying analytical techniques to the stored information, predictions of chemotherapeutic efficacy can be made. Methods of the invention allow for the skilled practitioner to accurately select an effective course of chemotherapy for a patients, thus reducing the risk of treatment-related trauma and resistance.

- [0040] In one aspect of the invention, a course of chemotherapy is selected based on results obtained from the phenotypic and genotypic assays. The present invention allows for the assessment of the likelihood of whether chemotherapeutic agents will be effective in treating a malignancy in a patient. A phenotypic assay in combination with a genotypic assay operates to minimize the risk of administering to a patient a chemotherapeutic agent or combinations of chemotherapeutic agents to which the tumor is resistant. In one aspect of the invention, chemotherapeutic agents or combinations of chemotherapeutic agents are selected for treatment where an effect on cellular phenotype is observed and the genotypic characteristics associated with resistance are not observed.
- 10 [0041] Chemotherapeutic agents that effect cellular phenotype are potential candidates for use in the patient. Known procedures that screen for chemotherapeutic agents are time-consuming and expensive. In one embodiment of the invention, chemotherapeutic agents that effect cellular phenotype and lack genetic changes associated with drug resistance are administered to the patient. In a further embodiment, genotypic characteristics observed in the
- 15 genetic assay undergo a comparative analysis to determine if such characteristics are associated with drug resistance. In another embodiment, the phenotypic and genotypic assays are performed in succession, thereby narrowing the scope of the genotypic comparative analysis, and reducing labor costs and associated expenses. In one aspect of the invention, when it is determined that a chemotherapeutic agent effects cellular phenotype and is not associated with
- 20 resistance to cells having the genotypic change, a patient is treated with the chemotherapeutic agent.

- [0042] The following examples provide further details of methods according to the invention. For purposes of exemplification, the following examples provide details of the use of methods of the present invention in colorectal cancer detection. Accordingly, while exemplified
- 25 in the following manner, the invention is not so limited and the skilled artisan will appreciate its wide range of application upon consideration thereof.

Example 1

- [0043] A patient was diagnosed with breast cancer and chemotherapeutic treatment was prescribed by the treating physician. A tumor biopsy of approximately 100 mg of non-necrotic,
- 30 non-contaminated tissue was harvested from the patient by surgical biopsy and transferred to a laboratory in a standard shipping container. Biopsy sample preparation proceeded as follows. Reagent grade ethanol was used to wipe down the surface of a Laminar flow hood. The tumor was then removed, under sterile conditions, from its shipping container, and cut into quarters with a sterile scalpel. Using sterile forceps, each undivided tissue quarter was then placed in 3

ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and minced by using two sterile scalpels in a scissor-like motion. After each tumor quarter was minced, the particles were plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask was
5 then labeled with the patient's code and the date of explantation. The explants were evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37° C incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks were placed in a 35° C, non-CO₂ incubator. Flasks were checked daily for growth and contamination as the explants grew out into
10 a cell monolayer.

[0044] Following initiation of prime cell culture of the tumor specimen, cells were removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10
15 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10X magnification--only those cells which did not take up the trypan blue dye were
20 counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated, and the optimum concentration of cells in the medium was determined.

[0045] Accommodating the above calculations, additional cell aliquots from the 4 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell
25 suspension were delivered into the microtiter plates using Terasaki dispenser techniques. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0046] Twenty-four hours post-plating, the chemotherapeutic agent paclitaxel sold under the trademark TAXOL (Bristol-Myers Squibb Company) was applied to the wells in the microtiter plates. Three treatment rows in the plates (Rows 2, 3, and 4) were designed to have escalating
30 paclitaxel doses (1.0, 5.0, and 25 µM). Row 5 served as a control. The paclitaxel exposure time was two hours. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation can be observed. During this period, the growth inhibiting effect of paclitaxel was monitored by observing the percent of confluency of the cells. For each microtiter well, the percent of confluency of cultured cells was plotted as a function of time.

[0047] Since paclitaxel affected growth rate of the cultured cells, cells from the patient were subjected to genotypic analysis. DNA was isolated from cells of the patient and analyzed for single nucleotide genetic polymorphisms. Known genetic polymorphisms were identified in the DNA by conducting PCR reactions and sequencing a region of interest in the DNA. The DNA region of interest from the patient cells was compared to corresponding regions from known genetic banks and libraries (for example, GENBANK).

[0048] The phenotypic and genotypic assays were used in combination to determine that paclitaxel was an efficacious course of treatment for the patient. As a result, paclitaxel was administered to the patient.

10 **Example 2**

[0049] A patient was diagnosed with lung cancer and chemotherapeutic treatment was prescribed by the treating physician. A tumor biopsy of approximately 100 mg of non-necrotic, non-contaminated tissue was harvested from the patient by surgical biopsy and transferred to a laboratory in a standard shipping container. The biopsy sample was prepared as described in Example 1. Twenty-four hours post-plating, the chemotherapeutic agent carboplatin sold under the trademark PARAPLATIN (Bristol-Myers Squibb Company) was applied to the wells in the microtiter plates. The first three treatment rows in the plates (Rows 2, 3, and 4) were designed to have escalating carboplatin doses (50, 200, and 1000 μ M). Row 5 serves as a control. The carboplatin exposure time was two hours. The cells were allowed to incubate for another 72 hours so that inhibition of cell motility can be observed.

[0050] Cell motility was measured by calculating the distance a cell travels over time. Cells were monitored using a digital video-camera mounted on a phase-contrast light microscope. To maintain the growth medium at 35° C, the microscope was fitted with a heated slide stage. After the cultured cells were incubated with carboplatin, cell migration was recorded under 20X magnification. During this period, the motility inhibiting effect of carboplatin was documented by plotting the distance cells travel as a function of time. The distance cells travel was a determined using digital imaging techniques known in the art.

[0051] Since carboplatin affected cell motility in the tumor cells, the cells were subjected to genotypic analysis by comparing DNA from the cultured cells to known genetic banks and libraries. Known genetic polymorphisms were identified in the cultured cells by conducting PCR reactions and sequencing a region of interest in DNA isolated from the cultured cells. The DNA region of interest from the cultured cells was compared to corresponding regions from known genetic banks and libraries (for example, GENBANK).

[0052] Genetic characteristics observed in the genotypic assay were compared to a database of genetic characteristics that were known to be associated with resistance to carboplatin. The phenotypic and genotypic assays were used in combination to determine that carboplatin was an efficacious course of treatment for the patient. As a result, carboplatin was administered to the
5 patient.

[0053] While the invention has been shown and described with reference to specific preferred embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the following claims.

10

Claims

1 What is claimed is:

2 1. A method for assessing efficacy of a chemotherapeutic agent, the method comprising:
3 conducting an assay to determine whether a chemotherapeutic agent affects
4 cellular phenotype of a sample of tumor cells obtained from a patient;
5 determining whether said patient comprises a genetic characteristic associated
6 with resistance to said chemotherapeutic agent; and
7 assessing efficacy of said chemotherapeutic agent based upon results of said
8 conducting and detecting steps.

1 2. The method of claim 1, wherein said sample of cells in said conducting step comprise
2 malignant cells.

1 3. The method of claim 1, wherein said sample of cells in said conducting step comprise
2 abnormal proliferating cells.

1 4. The method of claim 1, wherein said cellular phenotype is cell growth rate.

1 5. The method of claim 1, wherein said genotypic change is a genetic polymorphism.

1 6. The method of claim 1, wherein said determining step comprises sequencing a portion of
2 the genome of cells from said patient.

1 7. The method of claim 1, wherein said determining step comprises comparing said
2 genotype characteristic to a database of genotype characteristics associated with resistance to
3 said chemotherapeutic agent.

1 8. A method for selecting a chemotherapeutic agent for treating a patient, the method
2 comprising:
3 conducting an assay to determine whether a chemotherapeutic agent effects
4 cellular phenotype of a sample of cells from a patient;
5 determining whether cells from said patient comprise a genotypic characteristic
6 associated with resistance to said chemotherapeutic; and

7 selecting said chemotherapeutic agent for treating said patient if said
8 chemotherapeutic agent effects cellular phenotype of said sample of cells in said
9 conducting step and if said cells in said determining step do not comprise a genotypic
10 characteristic associated with resistance to said chemotherapeutic agent.

1 9. The method of claim 8, wherein said sample of cells in said conducting step comprise
2 malignant cells.

1 10. The method of claim 8, wherein said sample of cells in said conducting step comprise
2 abnormal proliferating cells.

1 11. The method of claim 8, wherein said cellular phenotype is cell growth rate.

1 12. The method of claim 8, wherein said genotypic change is a genetic polymorphism.

1 13. The method of claim 8, wherein said determining step comprises sequencing a portion of
2 the genome of cells from said patient.

1 14. The method of claim 8, wherein said determining step comprises comparing said
2 genotype characteristics to a database of genotype characteristics associated with resistance to
3 said chemotherapeutic agent.

1 15. A method for assessing efficacy of a chemotherapeutic agent on malignant cells in a
2 patient, the method comprising:
3 exposing malignant cells from a patient to a chemotherapeutic agent;
4 conducting an assay to determine whether said chemotherapeutic agent effects
5 cellular phenotype of said malignant cells from said patient;
6 determining whether a sample of cells from said patient comprise a genotypic
7 characteristic associated with resistance to said chemotherapeutic agent; and
8 assessing efficacy of said chemotherapeutic agent on said malignant cells from
9 said patient based upon results of said conducting and detecting steps.

- 1 16. The method of claim 15, further comprising:
2 selecting said chemotherapeutic agent for treating said patient if said
3 chemotherapeutic agent effects cellular phenotype of said malignant cells in said
4 conducting step and if said cells in said determining step do not comprise a genotypic
5 characteristic associated with resistance to said chemotherapeutic agent.
- 1 17. The method of claim 15, wherein said malignant cells are obtained from a tumor
2 specimen from said patient.
- 1 18. The method of claim 15, wherein said cells in said determining step are obtained from a
2 blood sample from said patient.
- 1 19. The method of claim 15, wherein said cells in said determining step are obtained from a
2 buccal smear from said patient.
- 1 20. The method of claim 15, wherein said cellular phenotype is cell growth rate.
- 1 21. The method of claim 15, wherein said genotypic change is a genetic polymorphism.
- 1 22. The method of claim 15, wherein said determining step comprises sequencing a portion
2 of the genome of cells from said patient.
- 1 23. The method of claim 15, wherein said determining step comprises comparing said
2 genotype characteristics to a database of genotype characteristics associated with resistance to
3 said chemotherapeutic agent.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2003/032285

A. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA/

Authorized officer

Facsimile No.

Telephone No.

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

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RECEIVED

APR 29 2004

TESTA, HURWITZ & THIBAUT

Date of mailing (day/month/year)
29 April 2004 (29.04.2004)

Applicant's or agent's file reference
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IMPORTANT NOTICE

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10 October 2003 (10.10.2003)

Priority date (day/month/year)
10 October 2002 (10.10.2002)

Applicant

PRECISION THERAPEUTICS, INC.

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this notice:

AU, AZ, BY, CH, CN, CO, DZ, EP, HU, JP, KG, KP, KR, MD, MK, MZ, RU, TM

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE, AG, AL, AM, AP, AT, BA, BB, BG, BR, BZ, CA, CR, CU, CZ, DE, DK, DM, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, ID, IL, IN, IS, KE, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PG, PH, PL, PT, RO, SC, SD, SE, SG, SK, SL, SY, TJ, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this notice is a copy of the international application as published by the International Bureau on 29 April 2004 (29.04.2004) under No. WO 2004/035833

4. **TIME LIMITS for filing a demand for international preliminary examination and for entry into the national phase**

The applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be **30 MONTHS** from the priority date, not only in respect of any elected Office if a demand for international preliminary examination is filed before the expiration of **19 months** from the priority date, but also in respect of any designated Office, in the absence of filing of such demand, where Article 22(1) as modified with effect from 1 April 2002 applies in respect of that designated Office. For further details, see *PCT Gazette* No. 44/2001 of 1 November 2001, pages 19926, 19932 and 19934, as well as the *PCT Newsletter*, October and November 2001 and February 2002 issues.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain designated or elected Offices. For regular updates on the applicable time limits (20, 21, 30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

For filing a demand for international preliminary examination, see the *PCT Applicant's Guide*, Volume I/A, Chapter IX. Only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination (at present, all PCT Contracting States are bound by Chapter II).

It is the applicant's sole responsibility to monitor all these time limits.

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